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Research article

EVALUATION OF ANTIBACTERIAL ACTIVITY OF PLANTS *BALANITES AEGYPTIACA* DEL. AND *TYLOPHORA INDICA* MERR. AGAINST RESISTANT ORGANISMS ESPECIALLY THOSE HARBOURING *BLA* GENES

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ABSTRACT: Alcoholic and aqueous extracts of endangered medicinal plants *Balanites aegyptiaca* and *Tylophora indica* were analyzed for antibacterial potential against various gram positive and gram negative bacteria including resistant strains harbouring *bla* genes by agar well diffusion method. Alcoholic extracts of both the plants showed activity against wider range of tested bacteria as compared to aqueous extracts which showed limited antibacterial activity. The alcoholic extract of leaf of *Tylophora indica* showed good activity against gram negative bacteria and mild activity against those harbouring *bla* genes, whereas, the alcoholic extract of fruit of *Balanites aegyptiaca* showed excellent antibacterial activity against gram positive, gram negative bacteria as well as resistant bacteria harbouring *bla* genes. Minimum inhibitory concentrations (MIC) of the alcoholic extracts were determined by broth microdilution method. The MIC values of the alcoholic fruit extract of *B. aegyptiaca* against tested bacterial species ranged from 1.53 to 49.0 µg/ml and MIC of alcoholic leaf extract of *T. indica* ranged from 3.05 to 98.0 µg/ml. The present study leads to conclusion that extracts of *Balanites aegyptiaca* and *Tylophora indica* contain good antibacterial activity which can be used as novel antimicrobial compounds in the treatment of various infections showing resistance to treatment by currently used antimicrobial agents.

Key words: *Balanites aegyptiaca*; *Tylophora indica*; antibacterial activity; resistant bacteria harbouring *bla* genes.

INTRODUCTION

Infectious diseases represent an important cause of morbidity and mortality. Bacteria have evolved numerous defense mechanisms against antimicrobial agents and their resistance to old and newly produced drugs is on rise. This is due to indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases (Sangeetha.S, et. al., 2012).

One of the commonly used antibiotics for the treatment of infections caused by broad spectrum of gram-positive and gram-negative bacteria is Beta-lactam antibiotics such as penicillins, cephalosporins and carbapenems. Bacteria develop resistance to these antibiotics because of production of enzyme β-lactamases which hydrolyze the β-lactam ring present in the antibiotic, rendering it inactive. These enzymes are synthesized due to presence of β-lactamase genes (*bla* genes) located either on the chromosome or plasmid (extrachromosomal genetic material) of bacteria. Examples of such resistant organisms harbouring *bla* genes include methicillin-resistant *staphylococci* (MRSA), *pneumococci* resistant to penicillin and macrolides, as well as multidrug resistant gram-negative organisms (Bush.K, et.al., 1995; Philippon.A, et.al., 2002; Norrby.R.S, et.al., 2005). Incidents of epidemics due to drug resistant micro-organisms are now a common global problem. This problem has even worsen due to the global emergence of multidrug resistant bacterial strains which are increasingly limiting the effectiveness of currently used drugs and causing treatment failure of infections (Hancock.E.W, 2005). This situation has forced the researchers to search for new antimicrobial substance from various sources including medicinal plants (Erdogrul.O.T, 2002; Bandow.J.E, et.al., 2003).

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There are several reports in literature regarding the antimicrobial activity of crude extracts prepared from plants (El-Seedi. H.R, et. al., 2002; Duraipandiyan.V, et.al., 2006; Parekh.J and Chanda.S, 2007). Antimicrobials of plant origin have proved effective in the treatment of several infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Samy.R.P and Ignacimuthu.S, 2000). *Balanites aegyptiaca* Del. an endangered medicinal plant belongs to family Zygophyllaceae. It is also known as 'desert date' in English, 'Hingoli' in Hindi and 'Angavriksha' in Sanskrit. This plant is a small evergreen thorny tree found in drier parts of India, Nigeria, Ghana and Ivory Coast. It grows to 6-10 m in height, is highly resistant to stresses such as sandstorms and heat waves, and grows with minimal available moisture. The trees produce date-like fruits between March and October. It has been found that various parts of the *Balanites* tree have been used as folk medicine in many regions of Africa and Asia. Literatures have revealed that bark, unripe fruits, and leaves of this plant have anthelminthic, antifertility, purgative, antifeedant, antidiabetic, molluscicide, contraceptive and antidysenteric properties. Dried fruits of this plant are being used as abortifacient by local healers and the root has been indicated for the treatment of malaria, herpes zoster, and venereal diseases (Kamel.M.S, et. al., 1991; Ibrahim.A.M, 1992; Rao.M.V, et.al., 1997; Mohamed.A.M, et.al., 2002; Gaur.K, et.al., 2008). *Tylophora indica* Merr. is an endangered medicinal plant indigenous to India and belongs to family Asclepiadaceae. It is also known as 'Indian ipecac' in English, 'Jangali pikvan' in Hindi and 'Anntmool' and 'Anthrapachaka' in Sanskrit. It is a dark copper coloured delicate creeper found growing wild in the sub-Himalayan region, central and peninsular India. It is also found in Ceylon, Malay island and Borneo. It is a perrineal, small, slender, much branched pubescent twining or climbing herbs or under shrubs. Flowers and fruits are produced between August-December. It has been used traditionally as folk medicine in certain regions of India for the treatment of bronchial asthma, inflammation, bronchitis, allergies, rheumatism and dermatitis. It is regarded as one of the best indigenous substitute for ipecacuanha, so it was considered as Indian ipecacuanha. The leaf extracts also act as anti tumour agents. It has also been used as an alternative blood purifier. The roots and leaf powder have been used in treatment of diarrhea, dysentery and intermittent fever. It was also found to be a good remedy in traditional medicine for psoriasis, anaphylaxis and leucopenia (Chitnis.M.P, et.al., 1972; Gore.K.V, et.al., 1980; Stephen.J and Vijayammal.P.L, 2000; Sangeetha.S, et.al., 2012).

The present study was done to determine the antibacterial activity of medicinal plants *B. aegyptiaca* and *T. indica* against both standard as well as clinical bacterial strains, with special reference to those harbouring resistant *bla* genes.

MATERIALS AND METHODS

Collection of plant materials

Fruit pulp of a 15 years old plant of *Balanites aegyptiaca* was obtained from Tissue culture Laboratory, Department of Botany, Gujarat University, Ahmedabad, Gujarat, and fresh leaves were collected from 6 years old plant of *Tylophora indica* grown in the Botanical garden, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh.

Preparation of plant extracts

Both aqueous and alcoholic extracts of the plants were tested for antibacterial activity. The extracts were prepared according to the method of Singh.I and Singh.V.P. (2000) with some modifications as described below. To prepare aqueous extracts, fresh fruit (15 g) of *B. aegyptiaca* and fresh leaves (15 g) of *T. indica* were taken and surface sterilized in 70% ethyl alcohol for 1 min and then washed 3 times with sterilized double distilled water (DDW). These were then grounded with sterilized pestle and mortar in 150 ml of DDW separately, then centrifuged at 5000 rpm for 15 min and the supernatant was filtered and taken as aqueous extract. Similarly, alcoholic extracts were prepared using 150 ml of 95% ethanol in place of DDW. The extracts were immediately used for experimentation.

Microorganisms Tested

The clinical bacterial strains included in our study were *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus vulgaris* isolated from clinical specimens in the Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh, India. In addition, resistant clinical isolates harbouring *bla* genes (identified by genotypic characterization done previously) were also included in the study. These isolates were *Escherichia coli* (*bla* $_{ampC}$), *Klebsiella* spp. (*bla* $_{CTX-M}$) and *Klebsiella* spp. (*bla* $_{SHV}$). The control strains tested were *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853), obtained from National Institute for Communicable Diseases (NICD), New Delhi, India. All the bacterial strains were grown on Blood agar or MacConkey agar plates at 37°C and maintained on nutrient and blood agar slants.

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Antimicrobial susceptibility testing

Antibacterial susceptibility testing was done by using agar well diffusion method (Akinpelu.D.A, 2001), with some modifications (Shahid.M, et.al., 2007) and performed as per guidelines of Clinical and Laboratory Standards Institute, formerly known as National Committee for Clinical Laboratory Standards (2000). Muellar-Hinton agar (M 173; HiMedia, India) plates and 5% sheep blood agar plates (for *Enterococci*) were used for determining antibacterial susceptibility testing. An inoculum of bacterial suspension containing 10^6 cfu/ml was used for inoculating the susceptibility plates. Two sets of Muellar-Hinton agar plates (one for aqueous extracts and the other for alcoholic extracts) were lawn cultured with the bacterial suspension with the help of sterile swabs. Wells of 5mm diameter were made in each plate using a sterile borer. Plant extracts (20µl) were poured in the wells using micropipette. Sterilized DDW and 95% ethanol (20µl each) were used as negative controls in the aqueous and alcoholic plates respectively, whereas, antibacterial agent gentamicin (500µg/20µl) was used as positive control. The plates were kept upright for 5-10 min until the solutions diffused into the medium and then incubated aerobically at 37°C for 24 hours. Later, the zone of inhibition was measured and recorded. All the experiments were performed in triplicate.

Determination of minimum inhibitory concentrations (MIC)

MIC was determined for alcoholic extracts by broth micro-dilution method performed according to Clinical and Laboratory Standards Institute (CLSI), formerly known as National Committee for Clinical Laboratory Standards, NCCLS (2000), with minor modifications (Shahid.M, et.al., 2007). Doubling dilutions of the extracts were prepared using RPMI-1640 broth (HiMedia, India) supplemented with 0.3g/L L-glutamine (HiMedia, India), 0.165 mol/L of 3-[N-morpholino] propanesulfonic acid (MOPS) buffer (HiMedia, India) and 0.01% of Dimethyl sulphoxide (DMSO) (Qualigens Fine Chemicals, India). The extracts were dissolved in DMSO, and further diluted 1:50 in RPMI-1640 medium, and each resulting solution was used for a doubling dilution series. Microtitre plates were prepared containing 100µl of undiluted extracts in the first well, followed by doubling dilutions of extracts from second well onwards. Standardized inoculum of each bacterial species was added to the dilution wells, including the first well. The final concentrations of the extracts ranged from 25×10^3µg/ml to 48×10^{-3}µg/ml. For each test there was a sterility control well containing alcoholic extract in RPMI-1640 broth plus DMSO and a growth control well containing bacterial suspension without alcoholic extract. The microtitre plates were incubated at 35 ± 2°C for 24 hours with their upper surface covered by sterile sealers. The lowest concentration of the extract that did not show any visible growth was considered MIC of the extract for that bacterial species. All the MIC experimentations were performed in duplicate.

Statistical analysis

All the experiments of antimicrobial susceptibility testing were performed in triplicate and the results were expressed as the mean ± standard error (SE). Data were statistically analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple analysis test (SPSS Software, Chicago, III, version 10). P values were calculated by one-sample T-test and $P < 0.05$ was taken as statistically significant.

RESULTS

Antibacterial activities of extracts of fruit of *B. aegyptiaca* and extracts of leaves of *T. indica* against the tested bacterial species are shown in Table 1 and 2 respectively. Amongst the negative controls used, sterilized DDW did not show any zone of inhibition, whereas, ethanol showed the zone of inhibition in the range of 0.00 to 8.67±0.33 mm. Positive control (gentamicin) showed the zone of inhibition in the range of 9.67±0.33 to 13.00±0.58 mm. Both the alcoholic extracts showed better antibacterial activity as compared to their aqueous extracts. Alcoholic fruit extract of *B. aegyptiaca* gave significant ($P<0.05$) antibacterial activity against both gram positive and gram negative bacteria (Table 1). It showed significant activity against *Staphylococcus aureus* (P=0.003), *Enterococcus faecalis* (P=0.012), *Escherichia coli* (P=0.005), *Klebsiella pneumoniae* (P=0.009), *Pseudomonas aeruginosa* (P=0.024) and *Proteus vulgaris* (P=0.017). It also showed significant ($P<0.05$) activity against most of the tested resistant bacteria harbouring *bla* genes except *Klebsiella* spp. (*bla* $_{SHV}$). The MIC values of the alcoholic fruit extract of *B. aegyptiaca* against tested bacterial species ranged from 1.53 to 49.0 µg/ml (Figure 1). Aqueous fruit extract of *B. aegyptiaca* showed significant ($P<0.05$) activity against *Staphylococcus aureus* (P=0.012) and *Escherichia coli* (P=0.017) only and no activity was detected against the resistant isolates (Table 1). The alcoholic leaf extract of *T. indica* showed good activity against most of the tested gram negative bacteria and no activity against tested gram positive bacteria (Table 2). It showed significant ($P<0.05$) activity against *Escherichia coli* (P=0.015), *Klebsiella pneumoniae* (P=0.012) and *Pseudomonas aeruginosa* (P=0.027).

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Amongst the tested gram negative bacteria harbouring *bla* genes it showed significant activity (P=0.038) only against *Escherichia coli* (*bla* $_{ampC}$).The MIC values of the alcoholic leaf extract of *T. indica* against tested bacterial species ranged from 3.05 to 98.0 µg/ml (Figure 2). The aqueous leaf extract of *T. indica* showed significant (P<0.05) antibacterial activity against *Escherichia coli* (P=0.027) and *Klebsiella pneumoniae* (P=0.038) only and no zone of inhibition was detected against gram positive bacteria and resistant bacteria harbouring *bla* genes (Table 2).

Table 1: Antibacterial activity of alcoholic and aqueous extracts of *Balanites aegyptiaca* against pathogenic bacteria including resistant isolates harbouring *bla* genes

Bacteria tested[β]	Zone of inhibition (mm) ± SE				
	Alcoholic fruit extract[Δ]	Aqueous fruit extract[Δ]	Ethanol[†] (negative control)	DDW[†] (negative control)	Gentamicin[£] (positive control)
Staphylococcus aureus	15.33±0.67^{a}	12.67±0.33^{c}	7.33±0.33^{d}	0.00±0.00^{a}	11.67±0.33^{bc}
Enterococcus faecalis	12.33±0.33^{cd}	0.00±0.00^{e}	7.33±0.33^{d}	0.00±0.00^{a}	9.67±0.33^{e}
Escherichia coli	12.33±0.33^{cd}	11.67±0.33^{d}	7.67±0.33^{c}	0.00±0.00^{a}	11.33±0.33^{c}
Klebsiella pneumoniae	11.67±0.33^{d}	0.00±0.00^{e}	7.33±0.33^{d}	0.00±0.00^{a}	10.67±0.33^{d}
Proteus vulgaris	11.33±0.33^{e}	0.00±0.00^{e}	8.33±0.33^{b}	0.00±0.00^{a}	10.67±0.33^{d}
Pseudomonas aeruginosa	11.33±0.33^{e}	0.00±0.00^{e}	7.33±0.33^{d}	0.00±0.00^{a}	10.67±0.33^{d}
Escherichia coli (*bla* $_{ampC}$)	12.33±0.33^{cd}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{a}	10.67±0.33^{d}
Klebsiella spp. (*bla* $_{CTX-M}$)	12.67±0.33^{c}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{a}	11.33±0.33^{c}
Klebsiella spp. (*bla* $_{SHV}$)	0.00±0.00^{f}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{a}	10.33±0.33^{de}
S. aureus ATCC 25923	14.67±0.33^{b}	13.33±0.33^{a}	8.67±0.33^{a}	0.00±0.00^{a}	13.00±0.58^{a}
E. coli ATCC 25922	13.67±0.33^{bc}	13.00±0.58^{b}	8.67±0.33^{a}	0.00±0.00^{a}	12.67±0.33^{b}
P. aeruginosa ATCC 27853	12.67±0.33^{c}	0.00±0.00^{e}	8.33±0.33^{b}	0.00±0.00^{a}	11.33±0.33^{c}

β = Figure within the parenthesis denotes the *bla* genes harboured by the respective clinical strains of gram-negative bacteria. † = concentration of negative controls used in test i.e. 20 µl each of 95% ethanol and DDW. Δ = concentration of extracts used in the test i.e. 2 mg / 20 µl. £ = concentration of gentamicin used in test i.e. 500 µg / 20 µl. Diameter of zone of inhibition is a mean of triplicates ± SE (mm). Differences were assessed statistically using one way ANOVA followed by Tukey's test. P<0.05 was considered as significant. The mean represented by same letter is not significantly different within the column.

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Table 2: Antibacterial activity of alcoholic and aqueous extracts of *Tylophora indica* against pathogenic bacteria including resistant isolates harbouring *bla* genes

Bacteria tested[β]	Zone of inhibition (mm) ± SE				
	Alcoholic leaf extract[Δ]	Aqueous leaf extract[Δ]	Ethanol[†] (negative control)	DDW[†] (negative control)	Gentamicin[£] (positive control)
Staphylococcus aureus	0.00±0.00[d]	0.00±0.00[d]	7.33±0.33[d]	0.00±0.00[a]	11.67±0.33[bc]
Enterococcus faecalis	0.00±0.00[d]	0.00±0.00[d]	7.33±0.33[d]	0.00±0.00[a]	9.67±0.33[e]
Escherichia coli	12.67±0.33[b]	12.33±0.33[b]	7.67±0.33[c]	0.00±0.00[a]	11.33±0.33[c]
Klebsiella pneumoniae	11.67±0.33[bc]	11.00±0.58[c]	7.33±0.33[d]	0.00±0.00[a]	10.67±0.33[d]
Proteus vulgaris	0.00±0.00[d]	0.00±0.00[d]	8.33±0.33[b]	0.00±0.00[a]	10.67±0.33[d]
Pseudomonas aeruginosa	11.33±0.33[c]	0.00±0.00[d]	7.33±0.33[d]	0.00±0.00[a]	10.67±0.33[d]
Escherichia coli (bla amp C)	11.67±0.33b[c]	0.00±0.00[d]	0.00±0.00[e]	0.00±0.00[a]	10.67±0.33[d]
Klebsiella spp. (bla CTX-M)	0.00±0.00[d]	0.00±0.00[d]	0.00±0.00[e]	0.00±0.00[a]	11.33±0.33[c]
Klebsiella spp. (bla SHV)	0.00±0.00[e]	0.00±0.00[d]	0.00±0.00[e]	0.00±0.00[a]	10.33±0.33[de]
S. aureus ATCC 25923	0.00±0.00[d]	0.00±0.00[d]	8.67±0.33[a]	0.00±0.00[a]	13.00±0.58[a]
E. coli ATCC 25922	13.00±0.58[a]	12.67±0.33[a]	8.67±0.33[a]	0.00±0.00[a]	12.67±0.33[b]
P. aeruginosa ATCC 27853	11.67±0.33[bc]	0.00±0.00[d]	8.33±0.33[b]	0.00±0.00[a]	11.33±0.33[c]

β = Figure within the parenthesis denotes the *bla* genes harboured by the respective clinical strains of gram-negative bacteria. † = concentration of negative controls used in test i.e. 20 µl each of 95% ethanol and DDW. Δ = concentration of extracts used in the test i.e. 2 mg / 20 µl. £ = concentration of gentamicin used in test i.e. 500 µg / 20 µl. Diameter of zone of inhibition is a mean of triplicates ± SE (mm). Differences were assessed statistically using one way ANOVA followed by Tukey's test. P<0.05 was considered as significant. The mean represented by same letter is not significantly different within the column.

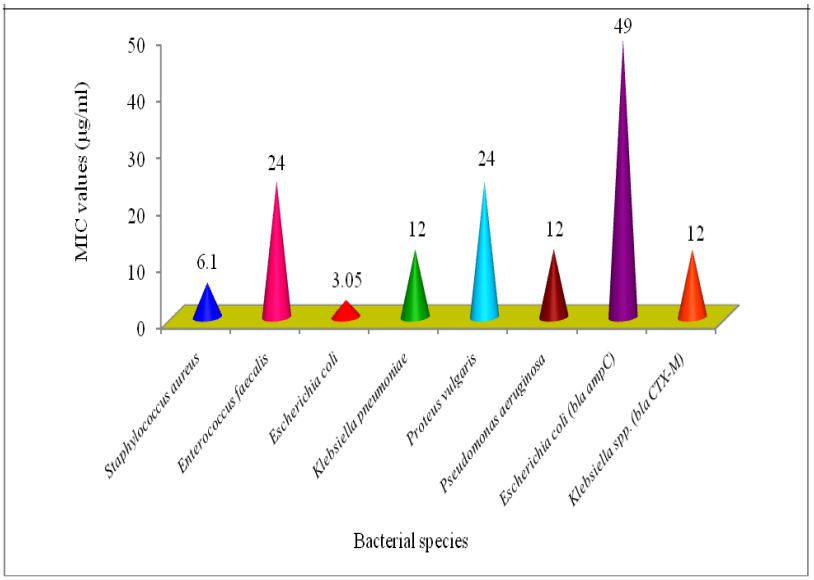

Figure 1: MIC determination of alcoholic fruit extract of *Balanites aegyptiaca* against tested pathogenic bacteria including resistant strains harbouring *bla* genes

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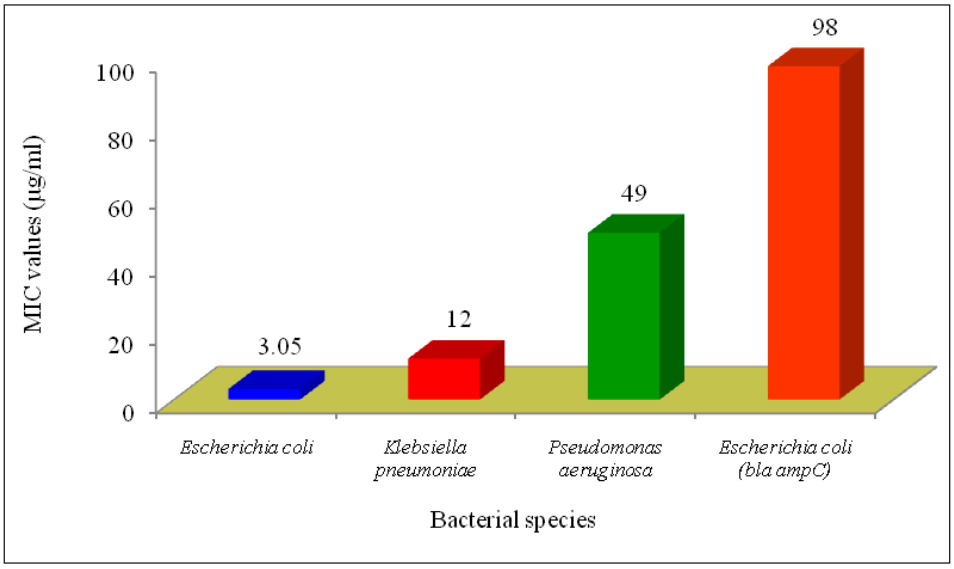

Figure 2: MIC determination of alcoholic fruit extract of *Tylophora indica* against tested pathogenic bacteria including resistant strains harbouring *bla* genes

DISCUSSION

The alcoholic extracts of *B. aegyptiaca* and *T. indica* gave good antibacterial activity as compared to their aqueous extracts. The alcoholic extract of fruit of *Balanites aegyptiaca* was found to be more efficient in controlling the growth of important bacterial pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus vulgaris* responsible for causing various diseases like wound infection, urinary tract infection, diarrhea and pneumonia. It was also found to be effective against most of the tested resistant bacteria harbouring *bla* genes. This shows the future prospect of the extracts of this plant to be used as novel antibacterial agents in the treatment of infections caused by these resistant organisms, which otherwise pose problems in treatment by the currently used antimicrobial drugs. Various studies have been done previously by different researchers to analyze the antibacterial potential of *B. aegyptiaca* (Doughari.J.H, et.al., 2007; Maregesi.S.M, et.al., 2008; Parekh.J and Chanda.S, 2008). They showed significant antibacterial activity of this plant against gram positive bacteria like *Staphylococcus aureus* and gram negative bacteria like *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, which supports our present research findings. The alcoholic leaf extract of *Tylophora indica* was found to be equally efficient in controlling the growth of most of the tested gram negative bacteria and significantly inhibiting the growth of resistant bacteria *Escherichia coli* (*bla* $_{ampC}$), but it did not show any activity against the tested gram positive bacteria. This finding of our study was found to be supported by an earlier done study which also showed no activity of this plant against gram positive bacteria (Parekh.J and Chanda.S, 2008). On the other hand, another study showed significant activity of this plant against *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris*, and less activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* (Reddy.B.U, 2010). Another study done by Sangeetha.S, et.al., (2012) showed significant activity of leaf extract of *T. indica* against *Staphylococcus aureus* only and no activity against *Escherichia coli* and *Pseudomonas aeruginosa*. These findings are in contrast with our study. This could be due to different concentrations of extracts used in their study as well as variation in active metabolites present in plant extracts derived from different places. Since, this is the first study analyzing the antibacterial potential of these plant extracts against resistant organisms harbouring *bla* genes, our findings could not be compared.

CONCLUSION

In nutshell, there is increasing incidence of antibiotic resistance in microorganisms to the commonly used antibiotics used for the treatment of infections. Though pharmaceutical industries have synthesized new drugs to combat this situation but due to side effects of these synthetic antibiotics search of alternative medicine derived from medicinal plants are now gaining popularity. Present study demonstrates that extracts of *B. aegyptiaca* and *T. indica* have remarkable antibacterial potential and thus can be used to derive novel antimicrobial agents for the treatment of various infections like diarrhea, wound infections, pneumonia and urinary tract infections, which otherwise pose problem of resistance to the currently used antimicrobial drugs.

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